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Anti-obesity Action of *Salix matsudana* Leaves (Part 2). Isolation of Anti-obesity Effectors from Polyphenol Fractions of *Salix matsudana*

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Previously, it was reported that polyphenol fractions prepared from the leaves of *Salix matsudana* reduced the elevation of the rat plasma triacylglycerol level at 3 and 4 h after oral administration of a lipid emulsion containing corn oil, at a dose of 570 mg/kg. Moreover, body weights at 2–9 weeks and the final parametrial adipose tissue weights were significantly lower in mice fed the high-fat diet with 5% polyphenol fractions of *S. matsudana* leaves than in those fed the high-fat diet alone. The polyphenol fractions of *S. matsudana* leaves also significantly reduced the hepatic total cholesterol content, which was elevated in mice fed the high-fat diet alone. In addition, the polyphenol fractions of *S. matsudana* leaves inhibited palmitic acid uptake into brush border membrane vesicles prepared from rat jejunum and α-amylase activity, and their fractions enhanced norepinephrine-induced lipolysis in fat cells. To clarify the active substances inhibiting the palmitic acid uptake into small intestinal brush border membrane, the α-amylase activity or enhancing the norepinephrine-induced lipolyis in fat cells, the isolation of the active substances from polyphenol fraction was attempted using the above three assay systems. Compounds 1, 2 and 3 were isolated from the polyphenol fractions and identified as apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside, respectively. Among three flavonoids, apigenin-7-*O*-D-glucoside inhibited α-amylase activity, and luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside inhibited palmitic acid uptake into small intestinal brush border membrane. Furthermore, three flavonoid glucosides enhanced norepinephrine-induced lipolysis in fat cells. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Salix matsudana; α-amylase activity; brush border membrane vesicles; flavonoids.

INTRODUCTION

In the previous report, it was found that polyphenol fractions of *S. matsudana* leaves prevented the increase of body weight with the increase of parametrial adipose tissue induced by feeding high-fat diet during the long term (Han *et al.*, 2003). Furthermore, the polyphenol fractions of *S. matsudana* leaves inhibited palmitic acid uptake into brush border membrane vesicles prepared from rat jejunum and α-amylase activity, and their fractions enhanced norepinephrine-induced lipolysis in fat cells (Han *et al.*, 2003). Therefore, the isolation of the anti-obesity effectors from polyphenol fractions of the leaves of *S. matsudana* was attempted using a lipolytic assay in rat adipocytes and an assay for inhibition of α-amylase activity *in vitro*, and inhibition of fatty acid uptake into brush border membrane vesicles prepared from rat jejunum.

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MATERIALS AND METHODS

Materials. The [1-^{14}C] palmitic acid was obtained from Du Pont NEN (England). Norepinephrine was purchased from Daiichi Pharmacy Co. (Tokyo, Japan). Collagenase (type IV) was purchased from Worthington Biochemical Co. (Freehold, NJ), and bovine serum albumin (BSA) was purchased from Wako Pure Chemical Co. (Osaka, Japan) and was extracted by the method of Chen (1967) to remove free fatty acids. Amylase was obtained from Sigma (St Louis, MO). Sephadex LH-20 was purchased from Pharmacia Biotech Co. (Sweden). Other chemicals were of reagent grade.

Plant material. The leaves of *Salix matsudana* were obtained from Jilin Sheng in China and voucher samples are stored at the Second Department of Medical Biochemistry, School of Medicine, Ehime University.

General experimental procedures. Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectra were measured at 300 MHz and 75 Hz, respectively, on a Brucker AC-300 (Germany).

Animals. Male Wistar King strain rats (5 weeks old) were purchased from Charles River Japan (Yokohama,

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Japan), and housed for 1 week in a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room. The animals were given free access to food and water. After adaptation to the lighting conditions for 1 week, the healthy animals were used in the present experiments. The Animal Studies Committee of Ehime University approved the experimental protocol.

Preparation of fat cells and measurement of norepinephrine-induced lipolysis in fat cells. Young male Wistar rats were killed by cervical dislocation, and their epididymal adipose tissue was quickly removed. Fat cells were isolated from the adipose tissue by the method of Rodbell (1964). The measurement of norepinphrine-induced lipolysis in fat cells was performed by the same methods described in a previous report (part 1) (Han *et al.*, 2003).

Measurement of α-amylase activity and lipid absorption by brush border membrane vesicles. The measurement of α-amylase activity and lipid absorption by brush border membrane vesicles was performed by the same methods described in a previous report (part 1) (Han *et al.*, 2003).

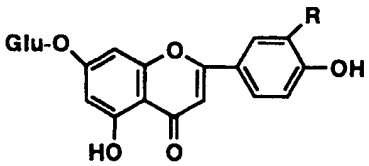
Isolation of lipolytic substances from polyphenol fractions augmenting norepinephrine-induced lipolytic activity. In the previous report, polyphenol fractions of *S. matsudana* enhanced norepinephrine-induced lipolysis in fat cells (Han *et al.*, 2003). Therefore, the polyphenol fractions (100 mg) were isolated by preparative thin layer chromatography with EtOH/*n*-BuOH/formic acid/H_2O (5:3:1:1) as eluant, to afford three active substances (compounds 1, 2 and 3) that enhanced norepinephrine-induced lipolysis. Compounds 1, 2 and 3 were identified as apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside, respectively, by direct comparison of the ^{1}H- and ^{13}C-NMR spectral data of an authentic sample (Fig. 1). The yield of compounds 1, 2 and 3 was 22.5, 13.1 and 27.8 mg, respectively.

Statistical analysis. The results are expressed as mean ± standard error (SEM). Data were analysed by one-way analysis of variance (ANOVA), and then differences in mean values among groups were analysed using Fisher's protected LSD multiple comparison test and were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

In a previous report, it was found that the 95% EtOH extract of *S. matsudana* leaves, the *n*-BuOH-soluble fraction separated from the 95% EtOH extract and polyphenol fractions prepared from *n*-BuOH-soluble fraction enhanced norepinephrine-induced lipolysis at a concentration of 1 mg/mL, but non-polyphenol glycoside fractions had no effect on norepinephrine-induced lipolysis (Han *et al.*, 2003). Therefore, the isolation of the active substances from the polyphenol fractions was attempted, yielding compounds 1, 2 and 3 and identified as apigenin-7-*O*-D-glucoside, luteolin-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside, respectively, by the comparison of their spectral data with authentic samples. Three compounds enhanced norepinephrine-induced lipolysis in fat cells (Fig. 2). These three compounds did not stimulate lipolysis in the absence of norepinephrine (data not shown). It has been reported that an α-amylase inhibitor from wheat flour prevented obesity through inhibition of digestion and absorption of carbohydrates (Yokota *et al.*, 1994; Lankisch *et al.*, 1998). Previously, it was reported that the polyphenol fractions inhibited amylase activity at concentrations of 250–5000 μg/mL. Among three isolated flavonoid glucosides, apigenin-7-*O*-D-glucoside inhibited the α-amylase activity at concentrations of 50–200 μg/mL, but luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside did not (Table 1). Three isolated flavonoid glucosides inhibited palmitic acid incorporation into small intestinal brush border membrane vesicles at 25–100 μg/mL (Table 2).

Although it has recently been reported that the leaves of *S. matsudana* have anti-obesity actions, the basis for this hearsay is unclear. Previously, experiments were designed to clarify whether high-fat diet-induced obesity in female mice was prevented by *S. matsudana* leaves, possibly due to the inhibition of intestinal absorption of dietary fat and carbohydrates (Han *et al.*, 2003). It was found that polyphenol fractions of *S. matsudana* leaves prevented the increases in body and parametrial adipose tissue weights in mice fed a high-fat diet containing 40% beef tallow for 9 weeks. It seems likely that the inhibitory effects of polyphenol fraction of *S. matudana* leaves on high-fat diet-induced obesity may be due to the inhibition of carbohydrate and lipid



Compound 1= apigenin-7-*O*-D-glucoside: R=H

Compound 2= luteolin-7-*O*-D-glucoside: R=OH

Compound 3= chrysoeriol-7-*O*-D-glucoside: R=OCH_3

Figure 1. Chemical structures of three flavonoid glucosides.

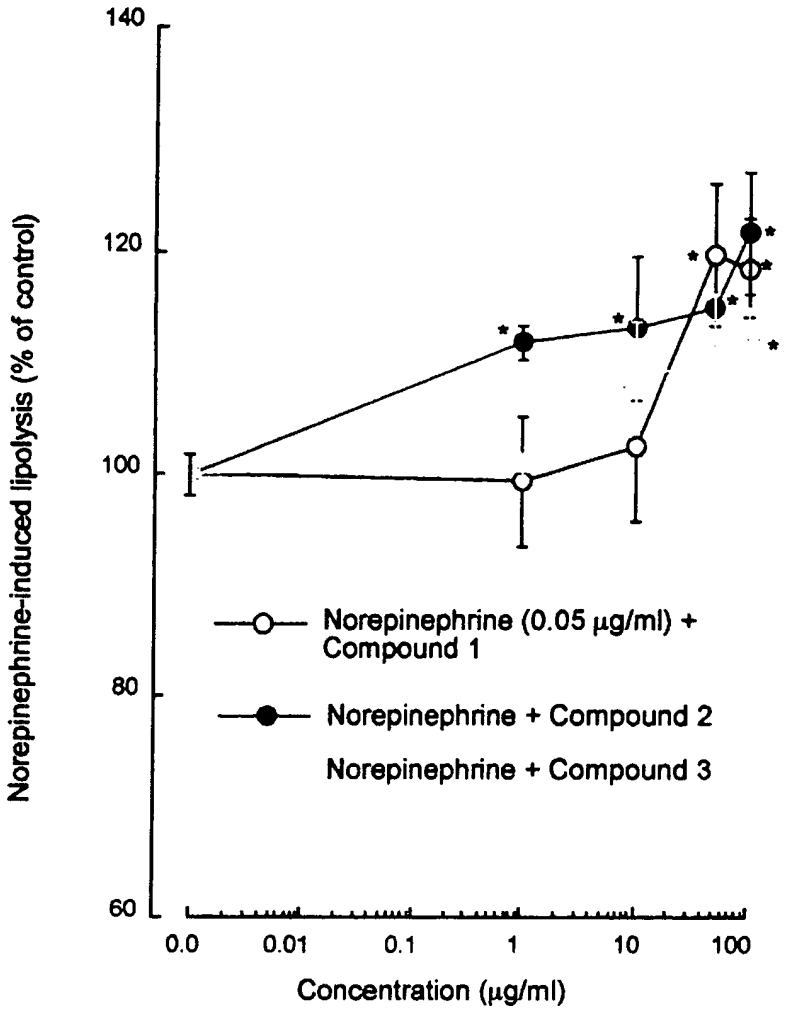


Figure 2. Effects of compounds 1, 2 and 3 isolated from polyphenol fractions of *S. matsudana* leaves on norepinephrine-induced lipolysis in fat cells. Results are expressed as the mean ± SEM, $n = 4$. The activity of norepinephrine-induced lipolysis is expressed as 100%. * $p < 0.05$, significantly different from norepinephrine alone.

Table 1. Effects of flavonoid glucosides isolated from polyphenol fractions of *S. matsudana* leaves on α-amylase activity

Addition (/mL reaction mixture)		α-Amylase activity (% of control)
None		100.0 ± 2.5
Apigenin-7-*O*-D-glucoside	(25 μg)	94.9 ± 0.1
	(50 μg)	87.3 ± 0.3*
	(100 μg)	74.6 ± 0.4*
	(200 μg)	50.6 ± 7.2*
Luteolin-7-*O*-D-glucoside	(25 μg)	99.8 ± 0.1
	(50 μg)	99.5 ± 0.1
	(100 μg)	97.8 ± 0.1
	(200 μg)	91.0 ± 0.3*
Chrysoeriol-7-*O*-D-glucoside	(25 μg)	101.2 ± 0.7
	(50 μg)	100.8 ± 0.01
	(100 μg)	99.2 ± 4.5
	(200 μg)	99.5 ± 4.1

Results are expressed as the mean ± SEM $n = 4$–8. * $p < 0.05$, significantly different from no addition (none).

Table 2. Effects of flavonoid glycosides isolated from polyphenol fractions of *S. matsudana* leaves on palmitic acid uptake into brush border membrane vesicles of rat jejunum

Addition (/mL reaction mixture)		Palmitic acid uptake to small intestinal brush border membrane (% of control)
None		100.0
Apigenin-7-*O*-D-glucoside	(25 μg)	56.1
	(50 μg)	50.0
	(100 μg)	36.9
Luteolin-7-*O*-D-glucoside	(25 μg)	66.8
	(50 μg)	48.2
	(100 μg)	22.2
Chrysoeriol-7-*O*-D-glucoside	(25 μg)	70.3
	(50 μg)	37.2
	(100 μg)	15.1

Results are expressed as the mean, $n = 2$.

absorption from the small intestine through the inhibition of α-amylase and palmitic acid uptake into small intestinal brush border membrane or accelerating fat mobilization through enhancing norepinephrine-induced lipolysis in fat cells (Han *et al.*, 2003). In the present study, apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside were isolated from the leaves of *S. matsudana* as potential activators of norepinephrine-induced lipolysis in fat cells. Kuppusamy and Das (1992) reported the effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. The mechanisms by which apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside stimulate norepinephrine-induced lipolysis are now under investigation. In addition, it was found that apigenin-7-*O*-D-glucoside isolated from *S. matsudana* leaves inhibited α-amylase activity, and that three flavonoid glucosides inhibited palmitic acid uptake into rat small intestinal brush border membrane vesicles. Therefore, it seems likely that the active substances in the anti-obesity action of the polyphenol fraction of *S. matsudana* leaves may be partly due to the flavonoid glycosides, such as apigenin-7-*O*-D-glucoside, luteolin-7-*O*-D-glucoside and chrysoeriol-7-*O*-D-glucoside. Experiments are now in progress to isolate the other active substance(s) from polyphenol fractions as well as three flavonoid glucosides.

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